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ORIGINAL ARTICLE

Effects of montelukast on M2-related cytokine and chemokine in M2 macrophages

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Abstract *Background/Purpose:* Asthma is a chronic airway inflammatory disease mediated by T-helper (Th)2 cells. Montelukast (trade name Singulair) is a cysteinyl leukotriene receptor antagonist used for asthma treatment. Mirroring Th1–Th2 polarization, two distinct states of macrophages have been recognized: the classically activated (M1) macrophages and the alternatively activated (M2) macrophages. M2 polarization is known to be a response to the Th2 cytokines; however, the effects of montelukast on M2 macrophages have not been well characterized. The aim of the present study was to investigate the effects of montelukast

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on the expression of cytokines and chemokines in M2-like macrophages, and to explore possible intracellular signaling pathways.
Methods: The human monocytic leukemia cell line THP-1 and human monocytes from healthy donors were cultured with interleukin-4 for M2 polarization, and then the cells were pretreated with or without montelukast before lipopolysaccharide (LPS) stimulation. Supernatants were collected to determine interleukin-10, I-309/CCL1, and MDC/CCL22 levels by enzyme-linked immunosorbent assay. Intracellular signaling was investigated using nuclear factor (NF)-κB inhibitors, mitogen-activated protein kinase (MAPK) inhibitors, and western blot analysis.
Results: LPS-induced interleukin-10 and I-309/CCL1 expression was significantly suppressed by montelukast in THP-1-derived and human monocyte-derived M2 macrophages after LPS stimulation. MDC/CCL22 expression was only significantly suppressed by montelukast in THP-1-derived M2 macrophages after 48 hours of incubation. In western blot analysis, montelukast was able to suppress LPS-induced MAPK-phospho-p38 and NF-κB-phospho-p65 expression.
Conclusion: Montelukast suppressed LPS-induced M2-related cytokines and chemokines in alternatively activated macrophages, and the effects might be mediated through the MAPK-p38 and NF-κB-p65 pathways.

Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by bronchial hyper-responsiveness with variable symptoms of recurring wheezing, coughing, and shortness of breath. Recently, the prevalence of asthma has grown in children, and it is associated with a large percentage of allergy-related comorbidity.[1–3] It is known that T-helper 2 (Th2) cells promote eosinophilic inflammation and mediate the pathogenesis of asthma.[4,5]

Macrophages are major airway immunocytes that are involved in lung immune homeostasis through phagocytosis and chemokine secretion in response to antigens. Regulation of phenotypes of pulmonary macrophages contributes to the pathogenesis of asthma.[6] Mirroring Th1—Th2 polarization, two distinct states of macrophages have been recognized: the classically activated (M1) macrophage phenotype and the alternatively activated (M2) macrophage phenotype.[7] While macrophages would typically be expected to polarize toward the M1 phenotype by Th1 cytokine interferon-γ, M2 polarization was originally discovered as a response to the Th2 cytokine interleukin (IL)-4.[8] M1 and M2 macrophages have distinct chemokine profiles. M1 macrophages express Th1 cell-attracting chemokines such as chemokine ligands CXCL9 and CXCL10.[9] M2 macrophages could be derived from resting macrophages by exposure to IL-4 or -13, and is characterized by expression of arginase-1, Ym-1, Fizz-1, mannose receptors, and scavenger receptors.[10] Chemokine ligands CCL1 (also known as I-309), CCL16, CCL17, CCL18, CCL22 (also known as MDC), and CCL24, and chemokine receptors CCR2, CXCR1, and CXCR2 are classified to characterize the M2 state.[11]

The regulatory roles of M2 macrophages in asthma remain controversial. Originally, M2 macrophages were reported to be negative regulators of immune response modulation, tissue repair, and restoration of homeostasis in the lung microenvironment.[12,13] However, recent studies found increased alternative activation of macrophages in the lungs of asthmatic patients relative to healthy control individuals, and excessive M2 macrophages may increase cell recruitment and mucus secretion, resulting in airway hyper-responsiveness.[14,15] Roles of M2 macrophages in asthma have not been well characterized.

Montelukast, trade name Singulair (Merck & Co., Inc., Kenilworth, NJ, USA), is a cysteinyl leukotriene receptor (cysLTR) antagonist that is used for the treatment of asthma.[16] In the airways and lungs, leukotriene is released by neutrophils, eosinophils, macrophages, epithelial cells, and vascular endothelial cells, and is involved in smooth muscle contraction, blood vessel dilatation, mucus secretion, and eosinophil recruitment.[17] It has been shown that the cysLTR plays a more important role than cysteinyl leukotriene in the pathogenesis of allergic airway inflammation.[18] Previous studies have demonstrated that montelukast significantly improves airway inflammation, pulmonary function, and symptom score in asthmatic wheezing infants compared with those in a placebo group[19] and reduces asthma exacerbations in children with asthma.[20,21]

Studies of the effects of leukotriene receptor antagonist on M2 macrophages are limited. Endotoxin, a lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, has been known to be a potent activator of macrophages and may induce exacerbation of asthma.[7] In the present study, we investigated the effects of montelukast on M2-related cytokine and chemokine activity in LPS-stimulated THP-1-derived and human monocyte-derived M2 macrophages and the possible intracellular mechanisms.

Methods

Cell preparation and reagents

The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI

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1640 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C and 5% CO_2 in a humidified incubator. Cells were centrifuged (1500 rpm, 5 min) and resuspended in fresh media in 24-well plates at a concentration of 5×10^5/mL for 24 hours before experimental use. THP-1 cells were cultured with phorbol 12-myristate 13-acetate (20 ng/mL) and IL-4 (20 ng/mL) for 3 days for M2 macrophage polarization, as described in a published study.[22] Mononuclear cells from peripheral blood samples collected from healthy donors were isolated by density-gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway), and the primary monocytes were isolated by magnetic bead sorting with anti-CD14 monoclonal antibody (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University Hospital, Kaohsiung, Taiwan and informed consent was obtained, according to the Declaration of Helsinki. The purity of the $CD14^+$ monocytes was $>$ 95%, and the $CD14^+$ monocytes were cultured with IL-4 (20 ng/mL) for 3 days for M2 macrophage polarization, as described in previous studies.[23,24]

THP-1-derived and human monocyte-derived M2 macrophages were pretreated with and without montelukast (10^{-7}—10^{-5}M; Sigma Chemical Co.) for 2 hours before LPS (0.2 μg/mL; *Escherichia coli*; Sigma Chemical Co.) stimulation. The mitogen-activated protein kinase (MAPK)-p38 inhibitor (SB203580), MAPK-c-Jun N-terminal kinase (JNK) inhibitor (SP600125), MAPK-extracellular signal-regulated kinase (ERK) inhibitor (PD98059), and nuclear factor (NF)-κB-p65 inhibitor (BAY117085) were used in the signal pathway analysis. All MAPK and NF-κB inhibitors were obtained from Sigma Chemical Company. The cell supernatant was collected after 24 hours and 48 hours of incubation.

Enzyme-linked immunosorbent assay

Concentrations of IL-10, MDC/CCL22, and I-309/CCL1 were measured by enzyme-linked immunosorbent assay kits following the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN, USA). Samples were measured in a Dynatech MRX plate reader at 450 nm and 540 nm using Revelation software (Dynatech Laboratories Ltd., Alexandria, VA, USA).

Cell viability

Montelukast was dissolved in dimethyl sulfoxide and diluted to the final concentrations with phosphate-buffered saline. Various concentrations of montelukast were incubated in 96-well plates for 24 hours. WST-1 activation solution was mixed and diluted at a ratio of 1:50. Then, 50 μL of the reaction solution was added to each well, and the plate was incubated for 5 hours. Cell viability was calculated by measuring the absorbance of the sample with an enzyme-linked immunosorbent assay reader (Bio-Rad Benchmark Plus microplate spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 470 nm and the reference wavelength of 650 nm. The mean value was used to assess the cell viability expressed as percent of control.

Western blotting analysis

After treatment for 2 hours with or without montelukast (10^{-6}—10^{-5}M), the cells were stimulated with LPS (0.2 μg/mL) for 30 minutes and lysed with equal volume of ice-cold 150 μL lysis buffer [20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM Ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, 1 μg/mL leupeptin, and protease inhibitor mixture]. After centrifugation at 13,000*g* for 15 minutes, equal amounts of cell lysates were transferred to a nitrocellulose membrane and blocked with Tris-buffered saline containing 1% nonfat dry milk and 0.1% Tris-buffered saline and Tween 20 for at least 2 hours. After washing three times with Tris-buffered saline and Tween 20, the membranes were incubated with primary antibodies (diluted 1:2000 in phosphate-buffered saline), including anti-MAPK (p38, ERK, and JNK; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-MAPK (phospho-p38, phospho-ERK, and phospho-JNK; Santa Cruz Biotechnology), anti-NF-κB (p65), and anti-phosphor-NF-κB (phosphor-p65; Cell Signaling Technology, Danvers, MA, USA), at 4°C overnight. The membranes were then incubated with secondary antibodies (diluted 1:5000 in phosphate-buffered saline). Immunoreactive bands were visualized using a chemiluminescence system (Amersham Pharmacia Biotech, Sunnyvale, CA, USA).

Statistical analyses

All data are presented as the mean ± standard deviation. All experiments were performed in at least triplicate, and differences between experimental and control groups were analyzed using a one-way analysis of variance test. A p value < 0.05 indicated a statistically significant difference between groups.

Results

Effects of montelukast on IL-10, I-309/CCL1, and MDC/CCL22 expression in THP-1-derived and human monocyte-derived M2 macrophages

To determine the effect of montelukast on cytokine and chemokine expression in M2 macrophages, M2 macrophages derived from THP-1 and human primary monocytes were pretreated with various concentrations of montelukast (10^{-7}—10^{-5}M) for 2 hours before LPS stimulation. The montelukast concentrations that we used in the present study are in the 10^{-7}—10^{-5}M concentration range, in agreement with cysteinyl leukotriene type 1 receptor antagonist activity that has been demonstrated in previous studies.[25,26] In THP-1-derived M2 macrophages, the results showed that montelukast (10^{-6}M and 10^{-5}M) significantly suppressed LPS-induced IL-10 after 24 hours of LPS stimulation (Fig. 1A), and montelukast (10^{-5}M) also significantly suppressed LPS-induced IL-10 after 48 hours of LPS stimulation (Fig. 1B). Montelukast (10^{-7}—10^{-5}M) significantly suppressed I-309/CCL1 in a dose-dependent manner after 24 hours of LPS stimulation (Fig. 1C), and also significantly

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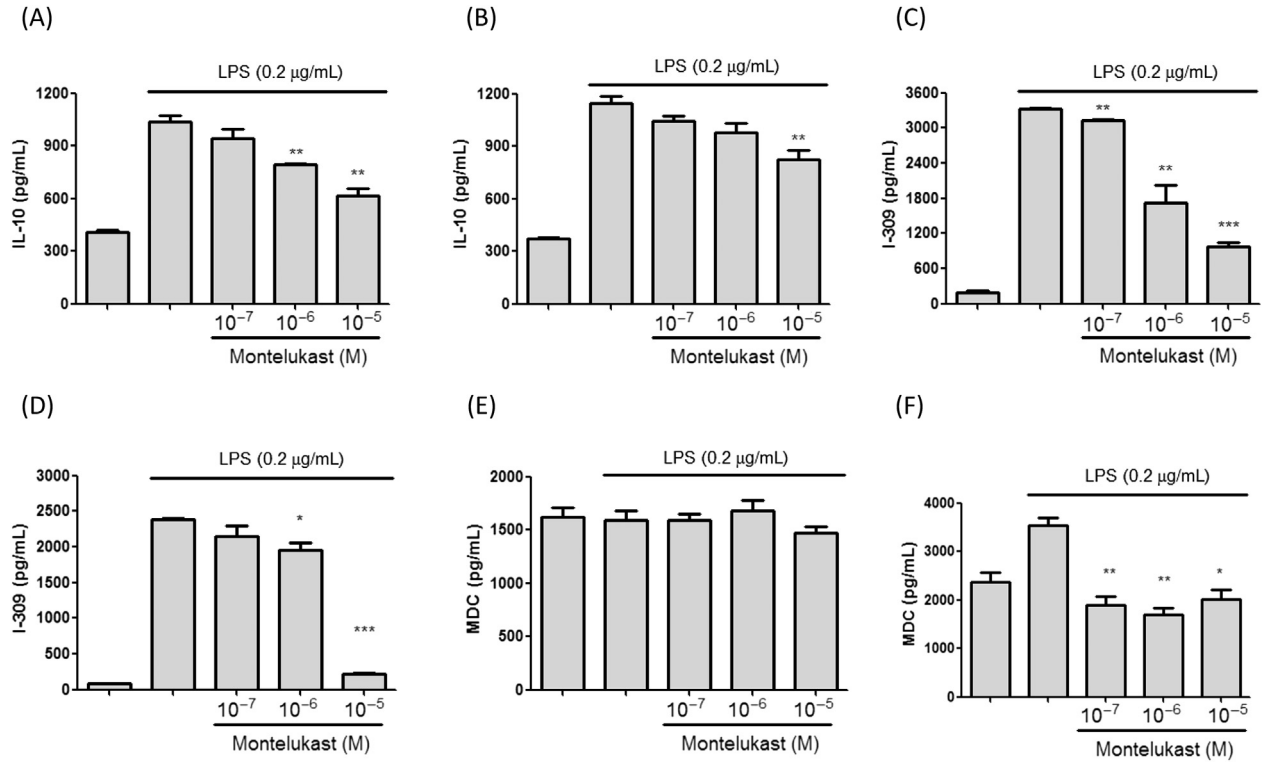

Figure 1. Effects of montelukast on IL-10, I-309/CCL1, and MDC/CCL22 expression in THP-1-derived M2 macrophages. THP-1-derived M2 macrophages were pretreated with various concentrations of montelukast (10^{-7}—10^{-5}M) for 2 hours before LPS (0.2 μg/mL) stimulation. Montelukast significantly suppressed LPS-induced IL-10 expression in THP-1-derived M2 macrophages after (A) 24 hours and (B) 48 hours of LPS stimulation. Montelukast significantly suppressed LPS-induced I-309/CCL1 expression in THP-1-derived M2 macrophages after (C) 24 hours and (D) 48 hours of LPS stimulation. (E) Pretreatment of montelukast (10^{-7}—10^{-5}M) had no effect on MDC/CCL22 expression after 24 hours of LPS stimulation. (F) MDC/CCL22 expression was significantly suppressed after 48 hours of LPS stimulation by montelukast. The bars represent the means ± standard deviations from three replicated experiments. * $p < 0.05$ compared with LPS alone. ** $p < 0.01$ compared with LPS alone. *** $p < 0.001$ compared with LPS alone. IL = interleukin; LPS = lipopolysaccharide.

suppressed I-309/CCL1 expression at concentrations of 10^{-6}M and 10^{-5}M after 48 hours of LPS stimulation (Fig. 1D). Treatment with montelukast (10^{-7}—10^{-5}M) appeared to not suppress MDC/CCL22 expression after 24 hours (Fig. 1E), but to significantly suppress MDC/CCL22 expression after 48 hours of LPS stimulation (Fig. 1F) in THP-1-derived M2 macrophages. In human primary monocyte-derived M2 macrophages, montelukast (10^{-5}M) significantly suppressed IL-10 and I-309/CCL1 expression, but not MDC/CCL22 expression, after 24 hours of LPS stimulation (Fig. 2). We found that LPS-induced IL-10 and I-309/CCL1 expression was significantly suppressed by montelukast in both THP-1-derived and human monocyte-derived M2 macrophages after LPS stimulation. MDC/CCL22 expression was only significantly suppressed by montelukast in THP-1-derived M2 macrophages after 48 hours of incubation.

Montelukast had no cytotoxic effects on M2 macrophages

Owing to the observed suppressive effect of montelukast on LPS-induced IL-10, I-309/CCL1, and MDC/CCL22 expression, we next determined the cytotoxic effect of montelukast on THP-1-derived and human monocyte-derived M2 macrophages at the concentrations employed in the experiment (10^{-6}M and 10^{-5}M) by WST-1 assay. Montelukast had no significant inhibition of cell viability compared with the control group (dimethyl sulfoxide only) in THP-1-derived M2 macrophages after 24 hours and 48 hours of incubation (Fig. 3A and 3B). As shown in Fig. 3C, montelukast also had no cytotoxic effects on human monocyte-derived M2 macrophages.

MAPK and NF-κB pathways may be involved in LPS-induced IL-10, I-309/CCL1, and MDC/CCL22 production in THP-1-derived M2 macrophages

We used various inhibitors to explore the key signaling pathway for M2-associated IL-10, I-309/CCL1, and MDC/CCL22. Our data showed that IL-10 was inhibited by SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), PD98059 (ERK inhibitor) (Fig. 4A), and BAY117085 (p65 inhibitor; Fig. 4B). I-309/CCL1 was suppressed by the JNK inhibitor, ERK inhibitor (Fig. 4C), and p65 inhibitor (Fig. 4D). We previously reported that SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) could suppress LPS-induced MDC/CCL22 in THP-1 cells.[27] These data suggested that MAPK and NF-κB pathways were important for LPS-induced IL-10, I-309/CCL1, and MDC/CCL22 synthesis.

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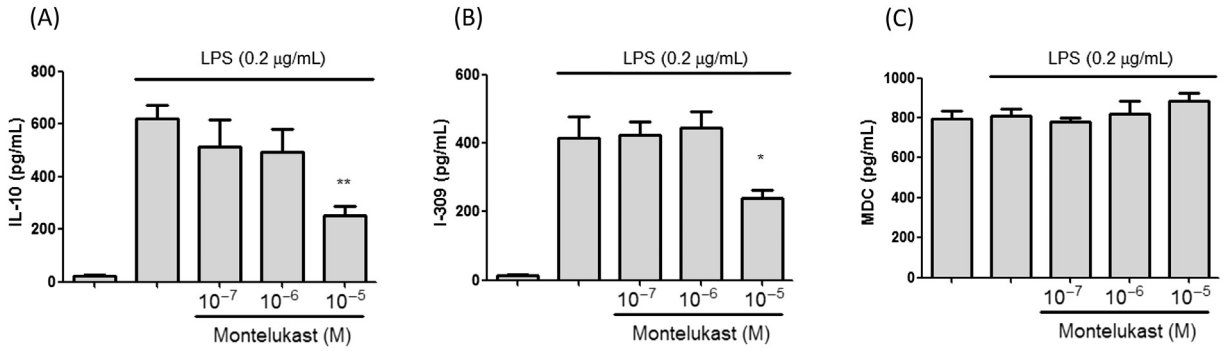

Figure 2. Effects of montelukast on IL-10, I-309/CCL1, and MDC/CCL22 expression in human monocyte-derived M2 macrophages. The human primary monocytes from healthy donors were cultured with IL-4 (20 ng/mL) for 3 days for M2 macrophage polarization and then were pretreated with montelukast (10^{-7}—10^{-5}M) for 2 hours before LPS (0.2 μg/mL) stimulation. Montelukast (10^{-5}M) significantly suppressed (A) IL-10 and (B) I-308/CCL1, but not (C) MDC expression after 24 hours of LPS stimulation in human monocyte-derived M2 macrophages. The bars represent the means ± standard deviations from three replicated experiments. * $p < 0.05$ compared with LPS alone. ** $p < 0.01$ compared with LPS alone. IL = interleukin; LPS = lipopolysaccharide.

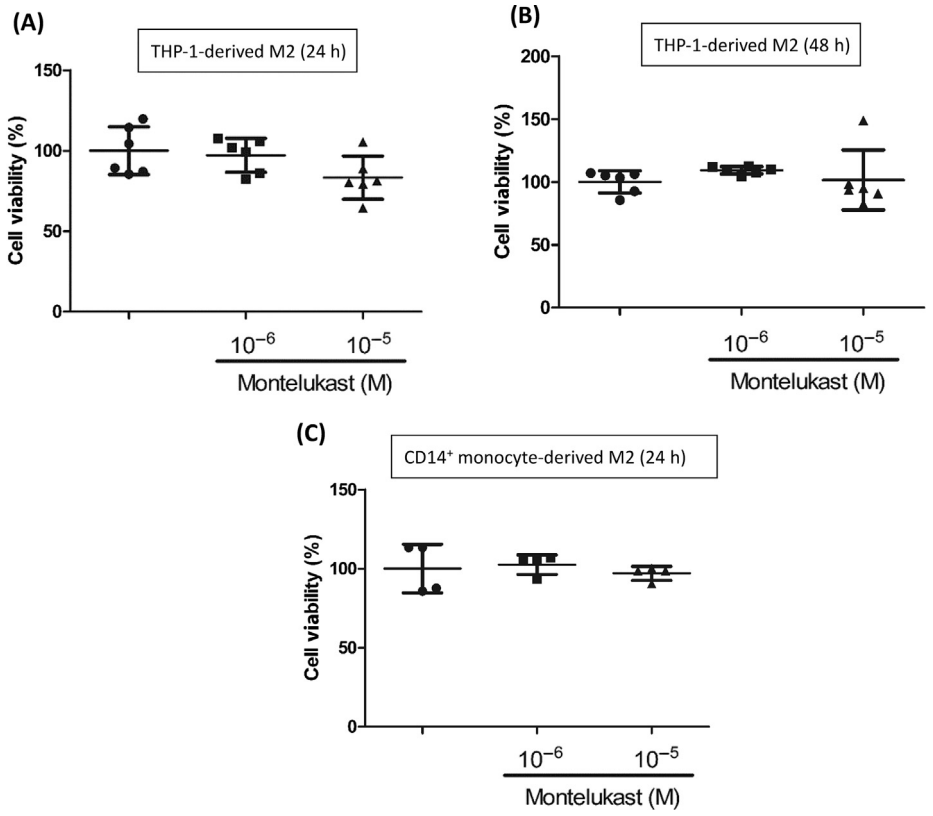

Figure 3. Effects of montelukast on cell viability of M2 macrophages. THP-1-derived M2 macrophages were pretreated with 10^{-6}M and 10^{-5}M montelukast in a 96-well plate for (A) 24 hours and (B) 48 hours. (C) Human monocyte-derived M2 macrophages were pretreated with 10^{-6}M and 10^{-5}M montelukast in a 96-well plate for 24 hours. Cell viability was determined by a WST-1 assay. Bars represent the means ± standard deviations of six individual experiments in THP-1-derived M2 macrophages and four individual experiments in human monocyte-derived M2 macrophages.

Montelukast downregulated LPS-induced chemokine expression in THP-1-derived M2 macrophages via MAPK-p38 and NF-κB pathways

We investigated the effects of montelukast on the phosphorylation of the MAPK family and NF-κB by western blot. Fig. 4 shows that montelukast suppressed the expression of LPS-induced phosphorylation of p38 (Fig. 5A), but not ERK (Fig. 5B) or JNK (Fig. 5C). Phosphorylation of p65 was also affected by montelukast, which indicated that NF-κB might be the cardinal signaling target for the suppressive effect of montelukast in THP-1-derived M2 macrophages (Fig. 5D).

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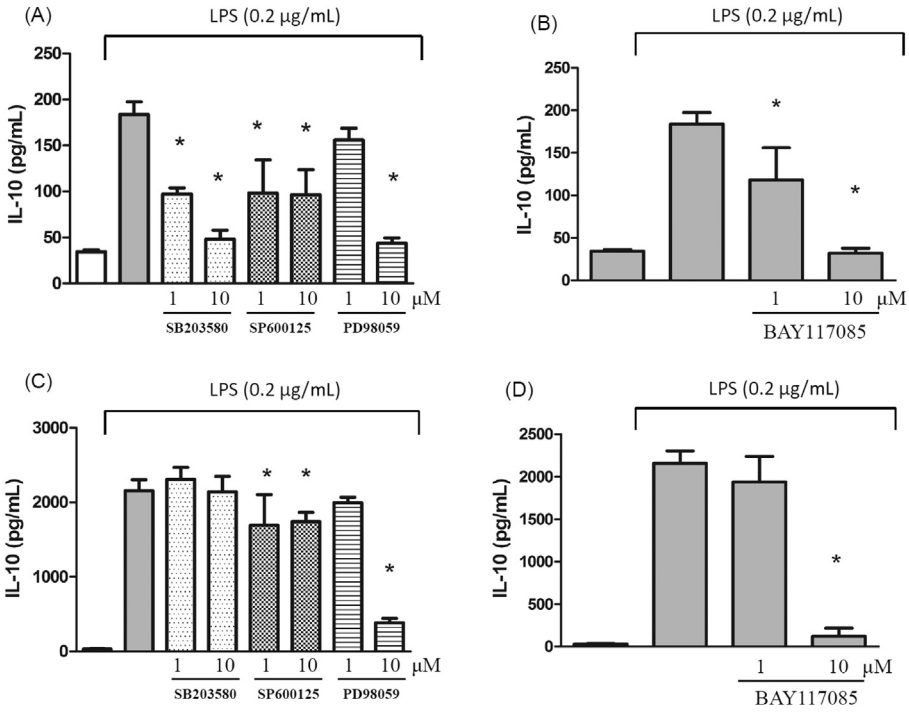

Figure 4. MAPK and NF-κB inhibitors suppressed LPS-induced IL-10 and I-309/CCL1 expression in THP-1-derived M2 macrophages. (A) MAPK inhibitors (1μM or 10μM), including SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and PD98059 (ERK inhibitor), and (B) BAY117085 [an NF-κB (p65) inhibitor] significantly suppressed LPS-induced IL-10 expression. I-309/CCL1 was significantly suppressed by (C) SP600125, PD98059, and (D) BAY117085. * $p < 0.05$ compared with LPS alone. ERK = extracellular signal-regulated kinase; IL = interleukin; JNK = c-Jun N-terminal kinase; LPS = lipopolysaccharide; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor-κB.

These data suggested that the effect of montelukast may involve the MAPK-p38 and NF-κB pathways.

Discussion

Montelukast is the most widely prescribed cysLTR antagonist for the treatment of allergic rhinitis and exercise- and aspirin-induced asthma. Montelukast is used to mediate the symptoms of these illnesses by blocking leukotriene receptors and preventing the activation of proinflammatory mediators in the airways. Montelukast is also used as add-on therapy in patients with asthma whose symptoms are poorly controlled by inhaled corticosteroid monotherapy.[28,29]

Macrophages have important roles in several chronic diseases, including asthma. The inflammatory response in asthma is characterized by the recruitment of Th2 lymphocytes, which produce several cytokines, including IL-4, IL-13, and IL-33, influencing airway macrophages toward alternative (M2) polarization.[10] In our study, montelukast was capable of suppressing IL-10 and I-309/CCL1 expression in LPS-stimulated THP-1-derived and human monocyte-derived M2 macrophages. This is the first study to investigate the effects of the cysLTR antagonist on M2-associated chemokine/cytokine activity in human M2-like macrophages. Wu et al[30] demonstrated that montelukast might exert its anti-inflammatory effect through suppression of Th2 cytokines. Their study showed that montelukast significantly reduced the concentrations of IL-4, -5, and -13, and also reduced the number of eosinophils in bronchoalveolar lavage samples. Stelmach et al[31,32] reported that increasing IL-10 levels were detected in the serum and supernatants of peripheral blood mononuclear cells (PBMCs) from asthmatic children under montelukast treatment. However, in our study, we found that LPS-induced IL-10 expression was significantly suppressed by montelukast in the supernatants of THP-1-derived and human monocyte-derived M2 macrophages from healthy donors. In humans, it has been demonstrated that IL-10 could be produced by Th1 and Th2 lymphocytes, cytotoxic T cells, mast cells, monocytes, B lymphocytes, keratinocytes, and eosinophils.[33,34] Immune cells *in vivo* always work in cross talk with other cells as a network. The different findings between our study and previous studies of the montelukast effects on IL-10 production might because the samples in those studies were from serum and supernatants of PBMCs, the levels would be the amount of IL-10 from various immune cells. Our study focused on the effects of montelukast in M2 macrophages. Melgert et al[15,35] reported that M2 macrophages were increased in both bronchoalveolar lavage fluid and airway tissue of asthmatic patients, and might aggravate allergic inflammation. It supported that M2 macrophages play an important role in the pathophysiology of asthma. Another possible reason for the different effects of montelukast on IL-10 levels might be the different donor sources. Stelmach et al[32] have demonstrated different responses of IL-10 concentration to montelukast under different allergic conditions. The enhanced effect of montelukast on IL-10 concentrations was found in

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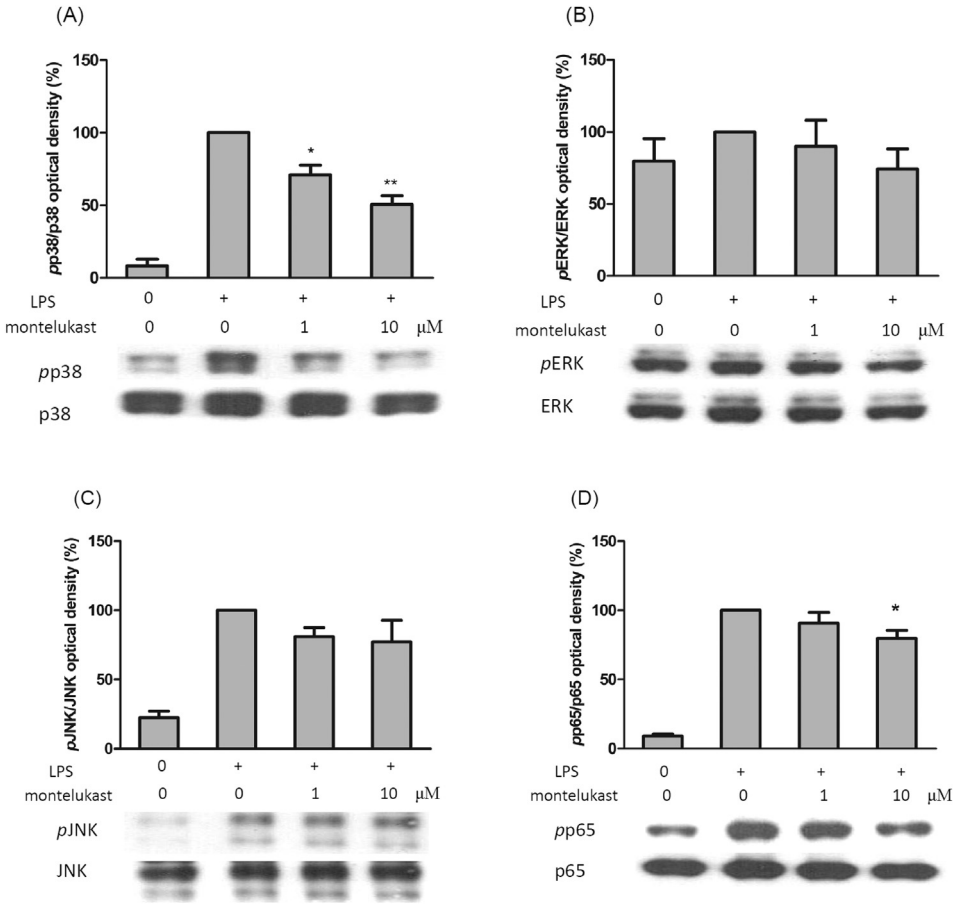

Figure 5. Western blot analysis of MAPK and NF-κB pathways. (A) Montelukast (1μM and 10μM) significantly downregulated LPS-induced phospho-p38 expression, but did not significantly downregulate LPS-induced expression of (B) phospho-ERK or (C) phospho-JNK in THP-1-derived M2 macrophages. (D) Phospho-p65 expression in THP-1-derived M2 macrophages was suppressed by montelukast at 10μM. The standard deviation of the optical density data refers to three independent experiments, and one experiment representative of the three is shown. * $p < 0.05$ compared with LPS alone. ** $p < 0.01$ compared with LPS alone. ERK = extracellular signal-regulated kinase; IL = interleukin; JNK = c-Jun N-terminal kinase; LPS = lipopolysaccharide; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor-κB.

supernatants from sensitizing allergen stimulation PBMC culture but not in unstimulated PBMC culture, and montelukast also had no effect on IL-10 concentrations in supernatants of children without clinical manifestation of asthma. Our study measured the LPS-induced IL-10 levels in the supernatants of THP-1-derived and human monocyte-derived M2 macrophages from healthy donors instead of asthmatic patients. Heterogeneity of IL-10 responsiveness to montelukast in M2 macrophages derived from healthy donors and asthmatic patients should be considered.

Montelukast exerts anti-inflammatory action through cysLTR-independent mechanisms, as well as via direct blockade of cysLTR.[36] MAPK signal pathways are important for many processes in immune responses, but the role of MAPK in macrophages is less characterized.[37] In this study, the p38, JNK, and ERK inhibitors reversed the LPS-induced IL-10 expression in THP-1-derived M2 macrophages. Western blot analysis further showed that LPS-stimulated phospho-p38 was significantly depressed under montelukast pretreatment in a dose-dependent manner, which was complementary to the inhibitor assay. However, the p38 inhibitor did not suppress the expression of I-309 in THP-1-derived M2 macrophages in our results. This indicates that the effects of montelukast on chemokine I-309 may differ from those of IL-10, which was regulated directly by the MAPK-p38 pathway in THP-1-derived M2 macrophages.

It has been reported that montelukast can inhibit the activity of the transcription factor NF-κB in monocytes/macrophages, interfering with the generation of various proinflammatory proteins such as IL-8.[38,39] In our study, we found that the NF-κB inhibitor suppressed LPS-induced IL-10 and I-309 expression in THP-1-derived M2 macrophages, and western blot analysis showed that LPS-stimulated phospho-p65 expression was depressed with higher concentrations of montelukast during pretreatment. Our results demonstrated that montelukast suppresses M2-related cytokines and chemokines, including IL-10 and I-309, maybe by interfering with the NF-κB pathway.

In the present study, we provided the first evidence that montelukast could significantly suppress LPS-induced M2-related cytokines and chemokines, including IL-10, I-309/

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CCL1, and partial MDC/CCL22, in human M2 macrophages. We also demonstrated that the effects might be mediated through the MAPK-p38 and NF-κB pathways.

In conclusion, montelukast may exert anti-inflammatory effects not only by directly blocking leukotriene receptors, but also by inhibiting cytokine and chemokine expression in alternatively activated macrophages through the MAPK-p38 and NF-κB pathways.

Conflicts of interest

All authors declare no conflicts of interest.

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